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ORIGINAL ARTICLE

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Fate of Prominin-1 Expressing Dermal Papilla Cells during Homeostasis, Wound Healing and Wnt Activation

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Prominin-1/CD133 (Prom1) is expressed by fibroblasts in the dermal papilla (DP) of the hair follicle (HF). By examining endogenous Prom1 expression and expression of LacZ in the skin of *Prom1CreERLacZ* (*Prom1*$^{C\text{-}L}$) mice, in which a *CreER*^{T2}*-IRES-nuclear LacZ* cassette is knocked into the first ATG codon of *Prom1,* we confirmed that Prom1 is expressed in the DP of all developing HFs and also by postnatal anagen follicles. To analyze the fate of Prom1+ DP cells, we crossed *Prom1*$^{C\text{-}L}$ mice with *Rosa26-CAG flox/stop/flox tdTomato* reporter mice and applied 4-hydroxytamoxifen (4OHT) to back skin at postnatal day (P) 1 and P2. We detected tdTomato+ cells in ~50% of DPs. The proportion of labeled cells per DP increased between P5 and P63, while the total number of cells per DP declined. Following full thickness wounding, there was no migration of tdTomato-labeled cells out of the DP. When β-catenin was activated in Prom1+ DP cells there was an increase in the size of anagen and telogen DP, but the proportion of tdTomato-labeled cells did not increase. We conclude that Prom1+ DP cells do not contribute to dermal repair but are nevertheless capable of regulating DP size via β-catenin-mediated intercellular communication.

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INTRODUCTION

Prominin-1 (CD133) is a pentaspan transmembrane glycoprotein that interacts with cholesterol and is involved in organizing the topography of the plasma membrane (Irollo and Pirozzi, 2013). Prominin-1 (Prom1) was originally identified as a human hematopoietic stem cell marker (Wognum *et al.*, 2003) and, in mice, as a marker of embryonic neuroepithelial cells (Shmelkov *et al.*, 2005; Yang and Cotsarelis, 2010). Prom1 has subsequently been used as a marker to isolate stem cells from various tissues, including brain, intestine, pancreas, prostate, and kidney (Zhu *et al.*, 2009). In addition, Prom1 is reported to be a marker for cancer initiating cells in a range of tumors, including tumors of the nervous system (Singh *et al.*, 2003), colon, and small intestine (Neuzil *et al.*, 2007; Shmelkov *et al.*, 2008; Snippert *et al.*, 2009; Zhu *et al.*, 2009).

In mouse skin, Prom1 is expressed by a subpopulation of hair follicle (HF) bulge stem cells (Charruyer *et al.*, 2012) and by bone marrow-derived cells (Lin *et al.*, 2014). Prom1 has also been identified as a marker of the fibroblasts of the dermal papilla (DP), which control HF development and regulate the hair cycle (Ito *et al.*, 2007b; Driskell *et al.*, 2009, 2011). Unlike Sox2, which is selectively expressed in guard/awl and auchene follicles, Prom1 is expressed in all HF types (Driskell *et al.*, 2009). In addition, low levels of Prom1 expression are detected in the arrector pili muscle (Ito *et al.*, 2007b; Driskell *et al.*, 2009). Prom1+ fibroblasts can be expanded at clonal density in culture and have the capacity to induce HF formation when transplanted into mice (Ito *et al.*, 2007b; Driskell *et al.*, 2009). DP cells express Prom1 in a transient manner, with strong expression during morphogenesis and anagen stages of the HF cycle (Ito *et al.*, 2007b; Greco *et al.*, 2009).

Previous studies have shown that the DP contains a very stable cell population that undergoes little proliferation or apoptosis (Tobin *et al.*, 2003a, b). Nevertheless, in mouse skin the number of cells within the DP increases during the first week after birth and decreases as the follicles transition from late anagen to telogen (Tobin *et al.*, 2003b). The concept that this reflects migration of cells between the DP and the adjacent dermal sheath (DS) (Tobin *et al.*, 2003b) has received support from different lines of investigation, including the observation that irradiated, nondividing DP cells can still

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Abbreviations: DP, dermal papilla; DS, dermal sheath; HF, hair follicle; Prom1, prominin-1; 4OHT, 4-hydroxytamoxifen

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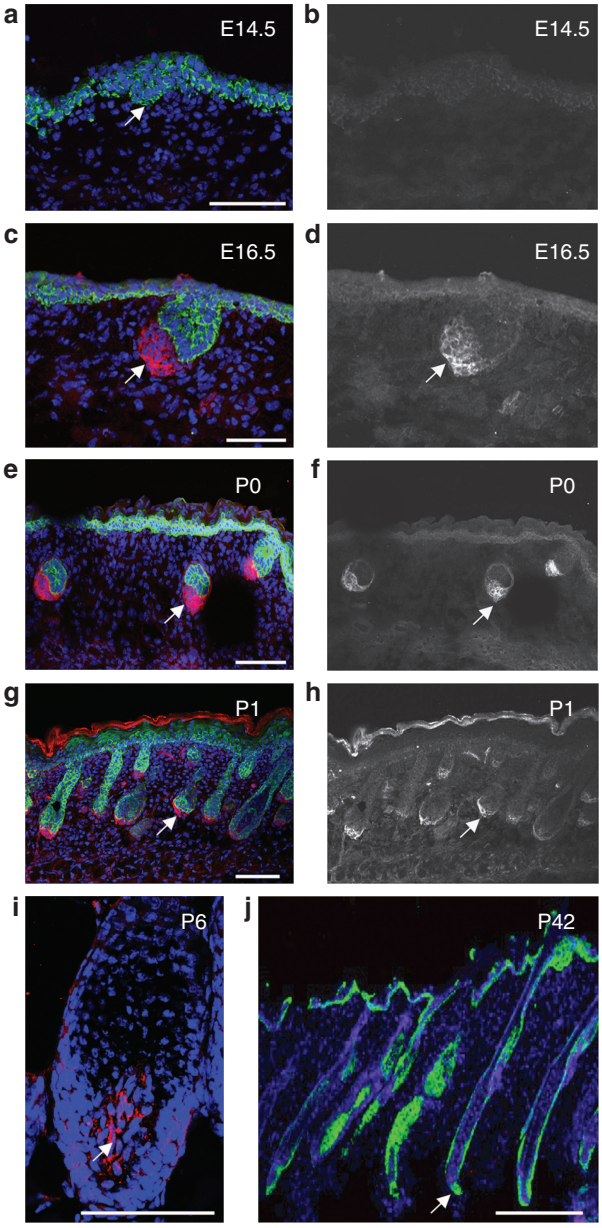

Figure 1. Endogenous Prominin-1 (Prom1) expression. (**a**, **c**, **e**, **g**, **i** and **j**) Dorsal skin of WT mice was collected at the stages indicated and labeled with antibodies to Prom1 (red) and keratin 14 (green), with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (blue). (**b**, **d**, **f** and **h**) are gray scale images of Prom1 labeling in (**a**, **c**, **e** and **g**), respectively. Arrows indicate dermal papillae. Scale bars = 100 μm. WT, wild type.

participate in HF reconstitution, provided that the DS cells are capable of dividing (Collins *et al.*, 2012). Cre-mediated lineage tracing using the DP marker Corin (Chi *et al.*, 2010) or the α-smooth muscle actin promoter to label progeny of the DS (Rahmani *et al.*, 2014) has further established that there is an interchange between cells of the DP and DS. The concept of cellular heterogeneity within the DP is supported by the observation that, whether formed during development or *de novo* in association with β-catenin-induced ectopic HF formation, each DP is polyclonal in origin (Collins *et al.*, 2012).

Taken together, the picture that is emerging of the DP is more dynamic than originally envisioned. This led us to use Prom1 expression as a marker to investigate the contribution of DP cells to the dermis under homeostatic conditions and when the skin is subject to perturbation by wounding or activation of Wnt signaling within the DP (Enshell-Seijffers *et al.*, 2010a, b).

RESULTS

Prom1/CD133 expression in the DP

We first examined endogenous Prom1 expression in mouse back skin at different embryonic and postnatal stages. At least three horizontal whole mount sections from each of three animals were examined per time point. We did not detect any Prom1+ cells at embryonic day (E) 14.5 (Figure 1a and b). However, at E16.5 Prom1 was expressed in the DP of all developing HF (Figure 1c and d) and continued to be expressed in all DP and in the lower DS during HF morphogenesis and the first postnatal anagen (Figure 1e–i). As reported previously (Ito *et al.*, 2007a; Greco *et al.*, 2009), Prom1 was not expressed in the DP in the catagen stage of the HF cycle (Figure 1j). We detected weak Prom1 expression in arrector pili muscle cells (data not shown). Although we did not detect Prom1 expression more widely in the dermis (Lin *et al.*, 2014) or in the HF bulge (Charruyer *et al.*, 2012), we cannot rule out the possibility that it is expressed in those sites, albeit at lower levels than in the DP.

For genetic lineage tracing (Kretzschmar and Watt, 2012), we used the previously described *Prom1CreER^{T2}nLacZ* (*Prom1$^{C\text{-}L}$*) mouse line (Zhu *et al.*, 2009). In these mice a *CreER^{T2}-IRES-nuclear LacZ* cassette is knocked into the first ATG codon of *Prom1* (*Prom1$^{C\text{-}L}$*) to create a null allele. We examined LacZ expression in the skin of *Prom1$^{C\text{-}L}$* heterozygous embryos by whole embryo labeling (Figure 2). At least three embryos were examined per time point. There was no labeling of the back skin at E14.5 (Figure 2a and b), but at E16.5 we observed labeling of all developing HF and whiskers (Figure 2c and d). There was no labeling of wild-type embryos, which served as a negative control (Figure 2b and d).

In postnatal tissue, both in whole mounts and conventional sections, LacZ was expressed in the DP of all HF from P0 through to P6 (Figure 3a–j; observations based on at least three mice per time point). There was no LacZ expression in the epidermis, in any regions of the dermis or hypodermis outside the DP and lower DS. Therefore there was an excellent correlation between LacZ and endogenous Prom1 expression (Figure 1).

Lineage tracing of Prom1+ DP cells

To genetically trace DP fibroblasts and their progeny, the *Prom1$^{C\text{-}L}$* strain of mice was crossed with *Rosa26-CAG flox/stop/flox tdTomato* reporter mice (Madisen *et al*, 2010) and 4-hydroxytamoxifen (4OHT) was applied topically to back skin at P1 and P2 (Figure 4a). TdTomato+ cells (red) were detected in the DP during HF morphogenesis (P5), anagen (P30), the catagen to telogen transition (P42), and late telogen (P63) (Figure 4b–i). Stages of the hair growth

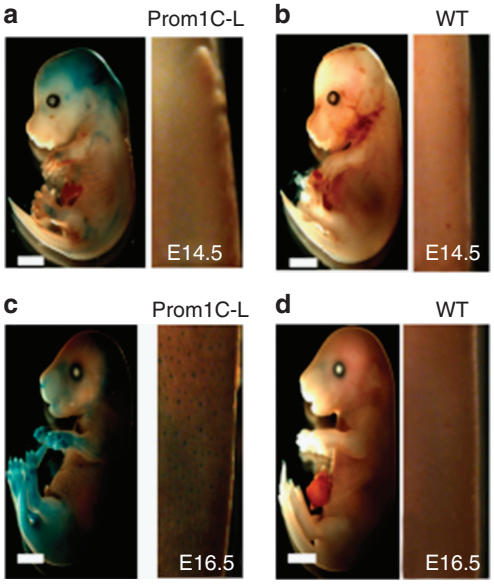

Figure 2. Expression of LacZ Prominin-1 (Prom1) reporter in mouse embryos. Whole mount labeling for β-galactosidase activity (blue) in Prom1$^{C\text{-}L}$ embryos (**a** and **c**) and WT littermates (**b** and **d**) at the developmental ages shown. Right hand panels: dorsal skin is shown at higher magnification. Scale bar = 1 mm. WT, wild type.

cycle were assigned according to the classification system of Muller-Rover *et al.*, 2001. In the absence of 4OHT, no tdTomato expression was detected in back skin (Supplementary Figure 1a, b online). We rarely detected tdTomato+ cells in the DS, which we ascribe to the low number of labeled cells overall and the high intensity of labeling within the DP.

At all time points examined, 50% of HFs had at least one tdTomato-labeled DP cell (Figure 4j). At P5 both Sox2+ (guard/awl/auchene) and Sox2 − (zigzag) follicles were labeled (Supplementary Figure 2a, b online). Quantitative analysis of all time points was carried out on zigzag HFs, which make up 80% of the follicles in back skin and are distinguishable from guard/awl auchene follicles based on their size (Driskell *et al.*, 2011). At least three mice were examined per time point. We quantitated a total of 25 DP per mouse by scoring all intact zigzag DP in 1–3 horizontal whole mount sections per mouse. We included dermal cup cells in the case of anagen follicles; cells in the DS were not scored. The proportion of tdTomato-labeled cells within each DP increased from 10% to ~40% between P5 and P42 (Figure 4k), while there was a decrease in the total number of DP cells (labeled and unlabeled) during the same period (Figure 4l; Supplementary Movies online). We did not detect any proliferative DP cells by EdU labeling (6 hour pulse) at any time point (150 DP analyzed per time point). In P5 back skin we detected a small number of Ki67+ DP cells in 3 out of 150 follicles analyzed (Supplementary Figure 3 online), leading us to conclude that there is little proliferation in the DP.

Labeled fibroblasts within the DP do not contribute to the wound bed

To discover whether Prom1-expressing DP fibroblasts and their progeny contributed to wound healing, we induced Cre recombination at P1 and P2 and then created full thickness circular wounds in the back skin of mice at P56. Back skin was collected and analyzed 8 days (Figure 5a–c) and 21 days (Figure 5d–f) after wounding. Skin from at least three mice per time point was analyzed. Although tdTomato-labeled cells were readily detected in the DP at the wound margins, no labeled cells were seen elsewhere in the epidermis or dermis (Figure 5). In all the sections examined the follicles adjacent to the wounds were Sox2 − . We conclude that Prom1+ DP cells associated with zigzag follicles do not contribute to other dermal compartments following wounding.

Overexpression of β-catenin in Prom1+ cells increases the size of the DP

β-catenin activity within the DP is required for HF maintenance (Tsai *et al.*, 2014) and regulates HF pigmentation and growth (Enshell-Seijffers *et al.*, 2010b) but does not influence HF types (Lei *et al.*, 2014). To examine the effects of β-catenin activation in Prom1+ DP fibroblasts, we generated triple mutant mice by intercrossing *Prom1*$^{C\text{-}L}$, *Rosa26-CAG flox/stop/flox tdTomato* reporter and *Ctnnb1* (Ex3)flox/+ mice (Harada *et al.*, 1999). Cre-mediated deletion of the third exon of *Ctnnb1* in *Ctnnb1*(Ex3)flox/+ mice results in constitutive β-catenin activation. Hence 4OHT treatment of triple mutant mice results in both tdTomato labeling of Prom1 + cells and β-catenin activation in those cells.

Mice were treated with 4OHT at P1 and P2 and the back skin was analyzed during adult anagen and telogen (Figure 6a–d) (Muller-Rover *et al.*, 2001). Nuclear β-catenin expression was detected in tdTomato+ DP cells (anagen and telogen) in triple mutant mice, confirming β-catenin activation (Supplementary Figure 4 online). In addition, some tdTomato − cells adjacent to tdTomato+ cells had nuclear β-catenin (Supplementary Figure 4 online), consistent with non-cell autonomous activation of Wnt signaling (Silva-Vargas *et al.*, 2005; Deschene *et al.*, 2014). In the absence of 4OHT, no tdTomato-positive cells were observed in back skin (Supplementary Figure 1c–d online).

The proportion of labeled DP cells in wild type and *Ctnnb1* (Ex3)flox/+ telogen follicles (Figure 6g) was determined in 30 labeled follicles (15 per mouse, two biological replicates) and was found to be similar to that of P30 DP (Figure 4k). The proportion of labeled cells in P65 anagen DP (a stage when the majority of follicles are in telogen) (Figure 6e) was somewhat lower, and resembled P5 DP (Figure 4k). In both anagen and telogen follicles of 4OHT treated skin, the total number of cells per DP was significantly increased on β-catenin activation (Figure 6f and h). However, the proportion of tdTomato+ cells was not affected (Figure 6e and g).

DISCUSSION

Our studies confirm that Prom1 is expressed in developing and postnatal DP, irrespective of HF type (Ito *et al.*, 2007b;

Driskell *et al.*, 2009), and that *Prom1*$^{C\text{-}L}$ reporter mice (Zhu *et al.*, 2009) can be used to label Prom1-expressing cells in the DP. In contrast to a previous report, we did not observe Prom1 expression in HF bulge stem cells (Charruyer *et al.*, 2012). This discrepancy could be due to differences in antibody specificity or sensitivity, and we have no reason to doubt that *Prom1* can be expressed in epidermal cells, particularly given the recent finding that in human skin a subset of epidermal cells in the hair placode express CD133 during early morphogenesis (Gay *et al.*, 2014). It is, nevertheless, important to note that in our study expression of the LacZ reporter in *Prom1*$^{C\text{-}L}$ mice was, like endogenous Prom1, primarily confined to the DP and DS. Therefore the lineage tracing experiments faithfully report the properties of cells in the DP that were expressing *Prom1* at P1-P2.

By inducing Cre expression shortly after birth, we were able to label Prom1-expressing cells in ~ 50% of DP and trace their progeny through a complete hair cycle. As reported previously (Tobin *et al.*, 2003a), the number of cells per DP declined between P5 (HF morphogenesis) and P63 (late telogen) (Figure 4l, Supplementary Movies online). During that time there was a significant increase in the proportion of tdTomato-labeled cells (Figure 4k), which occurred over a period when we did not detect any proliferative cells by EdU labeling. We therefore speculate that the increase in DP size occurs primarily via influx of cells into the DP. It is thought that some fibroblasts within the DP arise from the DS (Tobin *et al.*, 2003b; Chi *et al.*, 2010; Rahmani *et al.*, 2014). Our studies raise the possibility that Prom1+ DS cells are more likely to enter the DP than

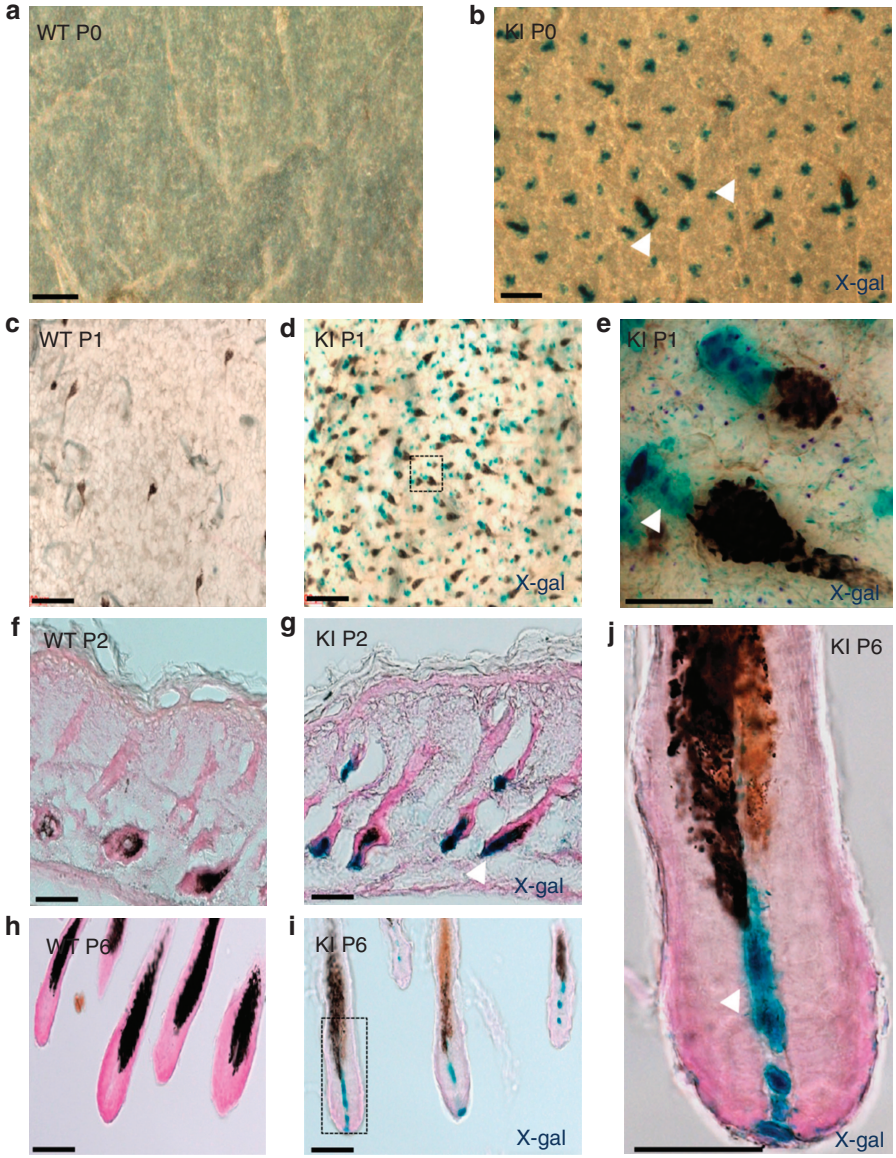

Figure 3. Expression of LacZ Prominin-1 reporter in postnatal skin. Whole mounts (**a**–**e**) and sections (**f**–**j**) of mouse back skin were labeled to detect β-galactosidase activity (blue). Sections were counterstained with haematoxylin (pink). Arrowheads indicate DP. Boxed region in (**i**) is shown at higher magnification in (**j**). Scale bars = 100 μm. DP, dermal papilla; KI, Prom1 C-L knock in; X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside; WT, wild type.

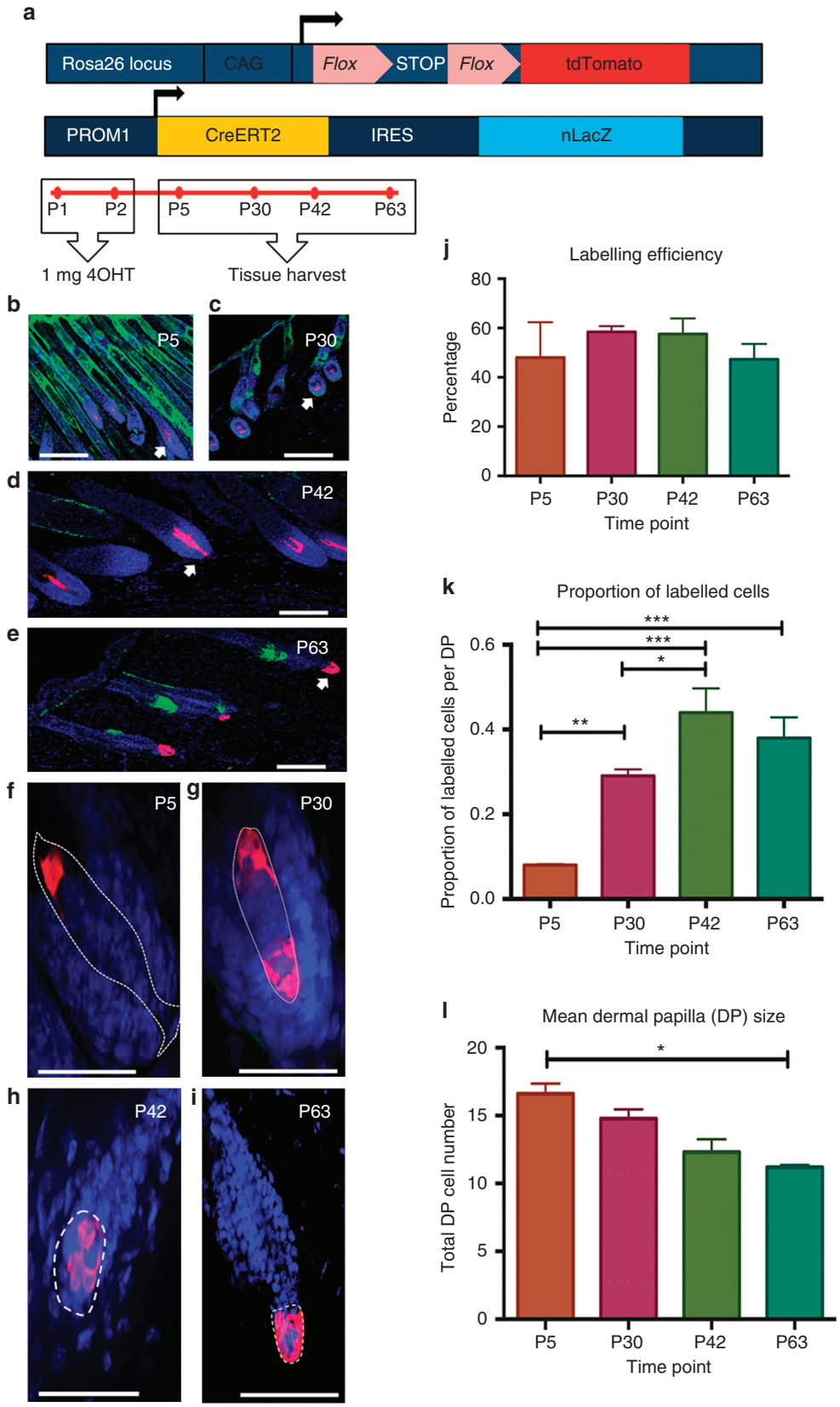

Figure 4. Lineage tracing of Prominin-1 (Prom1)-positive dermal papilla (DP) fibroblasts. (**a**) Schematic of labeling strategy. (**b**–**i**) Back skin of the stages indicated labeled for tdTomato (red) in back skin, keratin 14 (green; **b**–**e**) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Arrowheads indicate the DP. Dashed lines demarcate the DP, including the dermal cup (**f**). Scale bars = 100μm. (**j**–**l**) Quantitation of % DP labeled (**j**), proportion of tdTomato-positive cells per DP (**k**) and total number of cells per DP (**l**) at the stages indicated. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. Error bars represent mean ± SEM.

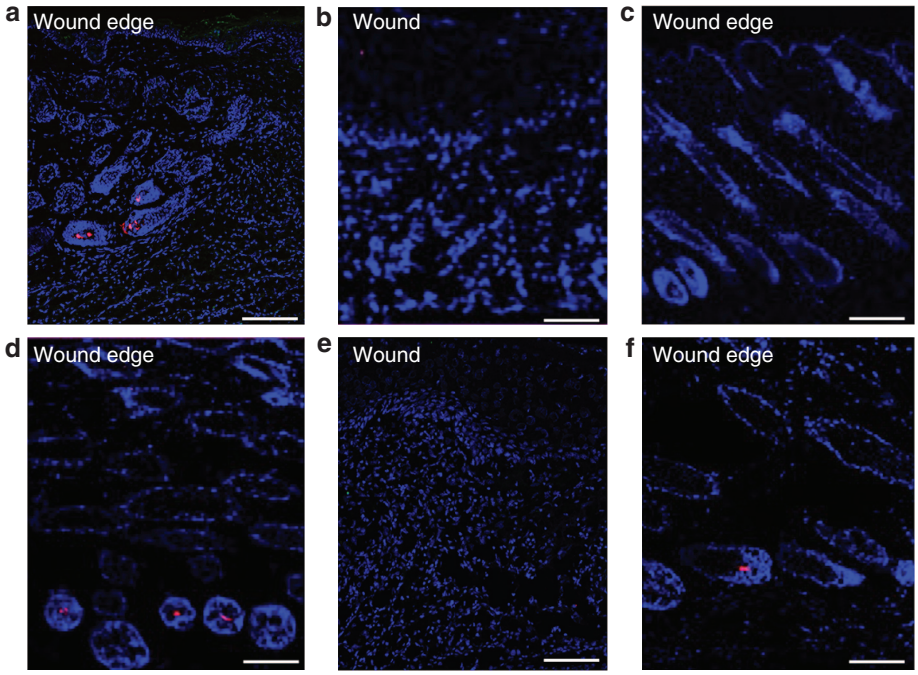

Figure 5. Lineage tracing of Prominin-1-positive dermal papilla fibroblasts following wounding. Wound edges (**a**, **c**, **d** and **f**) and wound bed (**b** and **e**) were analyzed on day 8 (**a**–**c**) and day 21 (**d**–**f**) after an 8-mm-diameter full thickness wound was made on the back. TdTomato expression (red) and nuclear 4′,6-diamidino-2-phenylindole (DAPI) labeling (blue) are shown. Scale bar = 100 μm.

Prom1 − cells, which is consistent with the recent report that Prom1 regulates the state of assembly of intercellular adhesive junctions (Gay *et al.*, 2014). Between the first and second hair cycles ~25% of zigzag follicles, corresponding to those with the highest number of DP cells, convert to awl/auchene follicles (Chi *et al.*, 2013) and this could also contribute to the decline in DP cell number observed.

Various studies have demonstrated that DP or DS cells can contribute to skin wound repair (Jahoda and Reynolds, 2001). When rat follicle dermal cells are implanted into skin wounds they are incorporated into the new dermis and are not restricted to HFs (Gharzi *et al.*, 2003). A subpopulation of Prom1+ DP cells co-express Sox2 (Driskell *et al.*, 2009) and Prom1+/Sox2+ dermal cells expanded in culture contribute widely to the dermis in skin reconstitution assays (Driskell *et al.*, 2012). Lineage tracing of transplanted Sox2+ dermal cells has shown that they can migrate out of the HF into the surrounding tissue (Biernaskie *et al.*, 2009). However, some dermal Sox2+ cells are not in the DP, but rather nerve-terminal-associated neural crest precursor cells around the HF bulge and these are found scattered through the dermis during wound healing (Johnston *et al.*, 2013). Although tdTomato+Sox2+ cells were detected in the DP (Supplementary Figure 2 online), no Sox2+ DP were present at the edges of the wounds we examined. In adult skin we saw no contribution of Prom1+/tdTomato+Sox2 − cells (associated with zigzag hairs) to wound healing. It therefore seems likely that contribution to dermal repair is not a ubiquitous property of DP cells, but rather confined to a subset of cells that are Sox2+.

There is extensive evidence that β-catenin signaling in the DP has profound effects on skin homeostasis. Inactivation of β-catenin in the DP of fully developed HFs results in premature induction of catagen and prevents HF regeneration (Enshell-Seijffers *et al.*, 2010a). Ablation of β-catenin specifically in DP cells at E14.5 during the first wave of guard hair formation leads to a reduction in guard hair numbers and loss of whisker follicles (Tsai *et al.*, 2014). A feature of β-catenin signaling in the skin is that it acts both cell autonomously and non-cell autonomously (Silva-Vargas *et al.*, 2005; Deschene *et al.*, 2014). In the DP loss and gain of function of β-catenin regulates hair pigmentation, acting on neighboring keratinocytes and melanocytes (Enshell-Seijffers *et al.*, 2010b).

We found that stabilization of β-catenin in Prom1-expressing DP cells results in an increase in the size of anagen and telogen DP (Figure 6f and h), without affecting the proportion of tdTomato+ cells (Figure 6e and g). This is consistent with non-cell autonomous signaling within the DP. However, the underlying mechanism remains to be identified. By Ki67 labeling we could detect very occasional proliferative cells in the DP (Supplementary Figure 3 online), but their incidence was not increased on β-catenin activation. Nevertheless, given the very low rate of proliferation in the DP, we cannot rule out the possibility that proliferation is increased when β-catenin is activated. We speculate that cell migration into the DP could account for the β-catenin-induced increase in DP size, as suggested for the changes that take place during the normal hair growth cycle.

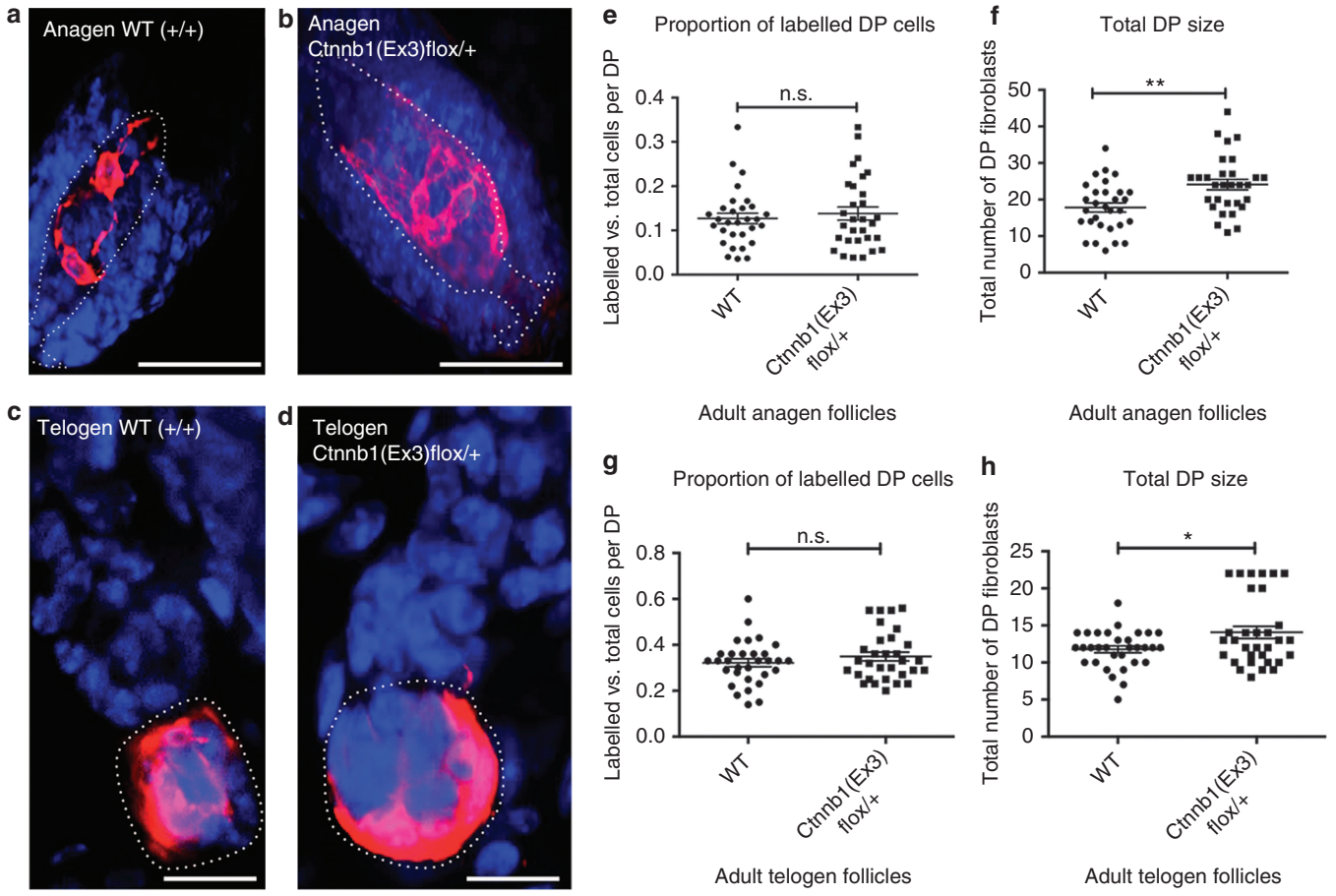

Figure 6. Effects of sustained β-catenin activation in the dermal papilla (DP). Prom1-positive cells were labeled with tdTomato, and anagen (P65) and telogen DP of WT and Ctnnb1(Ex3)flox/+ hair follicles were examined. Representative DP are shown in (**a**–**d**) with 4′,6-diamidino-2-phenylindole (DAPI) counterstain (blue). Dashed lines demarcate the DP. Scale bars = 100 μm. (**e**–**h**) Quantitation of the proportion of tdTomato-labeled cells per DP (**e** and **g**) and total DP size (**f** and **h**) in adult anagen (P65) (**e** and **f**) and telogen (**g** and **h**) follicles. $*P \leq 0.05$, $**P \leq 0.01$. Error bars represent mean ± SEM (two biological replicates per condition). WT, wild type.

In conclusion, our results highlight the dynamic nature of the DP. They further suggest that, not withstanding the heterogeneity of cells within individual DP, intercellular communication ensures that they function in a coordinated, tightly regulated manner.

MATERIALS AND METHODS

Transgenic mice

All procedures were carried out in accordance with the terms of a UK Government Home Office project license and subject to institutional ethical approval. All mice were maintained on a C57 Bl6/ CBA (F1) background. The *Prom1CreER^{T2}-IRES-nLacZ* (*Prom1$^{C\text{-}L}$*) knock-in mouse line (Zhu *et al.*, 2009) was used to analyze Prom1 expression by LacZ staining and also for lineage tracing. For lineage tracing, *Prom1$^{C\text{-}L}$* mice were bred to the *Rosa26-CAG flox/stop/flox tdTomato* gene trap strain of mice (Jackson Laboratory, Kent, UK, 007905) (LSL-tdTomato) (Madisen *et al.*, 2010) and Cre recombinase was activated by topical application of 1 mg 4OHT (Sigma-Aldrich, St Louis, MO) at postnatal day 1 (P1) and P2.

To activate β-catenin, Ctnnb1(Ex3)flox/+ mice (Harada *et al.*, 1999) were bred to homozygous *Rosa26-CAG flox/stop/flox tdTomato* reporter mice (Madisen *et al.*, 2010). Offspring heterozygous for both the conditional and the reporter allele were then bred again to the reporter mouse strain. Mice homozygous for the reporter and heterozygous for the conditional allele were then crossed with the *Prom1$^{C\text{-}L}$* heterozygous mouse line.

Wounding and lineage tracing

The *Prom1$^{C\text{-}L}$* mouse line (Zhu *et al.*, 2009) was crossed with the *Rosa26-CAG flox/stop/flox tdTomato* mouse line (Madisen *et al.*, 2010). Cre recombinase was activated by topical application of 1 mg 4OHT (Sigma-Aldrich) at P1 and P2. At P56, a single full thickness wound was made in the back skin of all littermates using an 8 mm diameter biopsy punch. The tissue was then collected at different time points. All analysis was carried out from a minimum of three biological replicates per time point.

Immunostaining and antibodies

For frozen sections the tissue was cryoproserved in Optimal Cutting Temperature (OCT) compound (VWR Chemicals, Lutterworth, UK #361603E) and sectioned at 5 μm thickness. Horizontal whole mounts were prepared as described previously (Driskell *et al.*,

2012, 2013). The following antibodies were used: Prom1 (1:50) (eBioscience, Hatfield, Ireland, UK, #14-1331-82), Keratin 14 (1:1000) (Covance, Cambridge, UK, #PRB-155P), Corin (1:100) (R&D systems, Minneapolis, MN, #AF2209), β-catenin (1:500) (Cell Signalling, Buckingham, UK, #8814S) and Ki67 (1:200) (Dako, Ely, UK, #M7249 clone TEC-3). All microscopy was performed on a Leica SP5 or Nikon A1 confocal microscope and images were analyzed using Image J (NIH).

X-gal staining

E14.5 and E16.5 embryos were rinsed with ice-cold PBS (supplemented with Mg^{++} and Ca^{++}). They were then fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) supplemented with 2 mM $MgCl_2$ (PBS-$MgCl_2$), 5 mM EGTA and 2% formalin for 15 minutes at room temperature. Fixed embryos were washed with 0.1 M phosphate buffer (pH 7.3) supplemented with PBS-$MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP40 (IGEPAL CA-630), two to three times for 30 minutes. The embryos were then incubated in PBS-$MgCl_2$ containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mM X-gal (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside) overnight at 37 °C to visualize β-galactosidase activity (Chhatriwala *et al.*, 2012). At least three biological replicates were analyzed per embryonic time point.

The same protocol was followed to label dermal whole mounts of P0 and P1 back skin. Additionally 5 μm thick cryosections were obtained from the back skin of mice at P2 and P6. For the cryosections, the same protocol was followed and the tissue had been fixed for 1 hour in 10% neutral buffered formalin before cryopreservation. LacZ-labeled embryos and whole mounts were imaged using a Zeiss ImagerM2 (Carl Zeiss, Mannheim, Germany). Back skin was analyzed from at least three biological replicates for each time point.

Quantitation of DP cell number

The number of cells per DP was counted manually in Z-stacks of 60 μm-thick horizontal whole mounts. By scanning the full thickness of each section it was possible to determine whether a given DP was fully captured within the whole mount (see Supplementary Movies online). Individual DP that were only partially contained within a whole mount were excluded from analysis. Guard/awl/auchene HFs were identified based on size and/or Sox2 labeling.

For the lineage tracing studies in Figure 4, back skin was analyzed from three biological replicates for each time point. Twenty-five follicles were analyzed from each mouse by scoring all adjacent zigzag follicles per section in two to three sections. By pooling data from three mice, a total of 75 follicles was analyzed per time point. To quantify changes in DP size as a result of β-catenin overexpression (Figure 6), back skin follicles were analyzed from two biological replicates and the results were pooled. Fifteen labeled follicles were analyzed from each biological replicate by scoring all adjacent zigzag follicles per section in two to three sections. By analyzing all adjacent labeled follicles we avoided a selection bias, as far as possible.

Statistical analysis

All graphs were generated using Microsoft Excel (2011) and GraphPad software (GraphPad Prism 6, La Jolla, CA). Data are represented as mean ± SEM. To analyze the slow expansion of fibroblasts within the DP over time, a one-way analysis of variance parametric test was performed for experiments, with $P<0.05$ considered statistically significant. To analyze the effect of overexpression of β-catenin in a subpopulation of cells within the DP, the D'Agostino-Pearson omnibus normality test was first carried out on the raw data. Since this revealed a normal distribution the unpaired *t*-test (with Welch's correction) was performed, with $P<0.05$ considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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